REMARKS/ARGUMENTS

Reconsideration and allowance of the present application based on the following remarks are respectfully requested.

Claims 23-25 and 27-33 stand rejected under 35 U.S.C. §103(a) as being unpatentable over Vitti *et al.* (Acta Med. Austriaca 23(1-2): 52-6, 1996) in view of Harlow *et al.* (Antibodies A laboratory Manual, Cold Spring Harbor Laboratory 1988, pages 556, 564-591), and Nicholson *et al.* (J. Mol. Endocrinol. 16(2): 159-70, 1996) or Morgenthaler *et al.* (J. Clin. Endocrinol. Metab. 81(2): 700-6, 1996). In addition, these claims further stand rejected under 35 U.S.C. §103(a) as unpatentable over US 5,614,363 in view of Vitti *et al.*, Harlow *et al.*, Nicholson *et al.* or Morgenthaler *et al.* The Applicants respectfully traverse these rejections for at least the following reasons.

The MPEP sets forth the requirement upon the Office to establish a *prima facie* case of obviousness as follows: [1]

> To establish a *prima facie* case of obviousness, three basic criteria must be met. First, there must be some suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the reference or to combine reference teachings. Second, there must be a reasonable expectation of success. Finally, the prior art reference (or references when combined) must teach or suggest all the claim limitations.

Although the Examiner admits that the presently claimed invention differs from Vitti *et al.* because "the recombinant human TSH receptor is immobilized to a solid support by a selective monoclonal antibody that recognizes only conformational epitopes of the human TSH receptor.."[2], it is nonetheless asserted that this deficiency is remedied by Harlow *et al.* "...because Harlow *et al.* teach that the advantages of antibody sandwich immunoassays are: it [sic] is rapid, easy, quantitative, and sensitive ..."[3] or by use of a specific antibody referenced Morgenthaler *et al* or Nicholson *et al.* The Applicants submit that there is no sufficient suggestion to combine the references cited above in the manner proposed by the

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[1] MPEP §2143.

[2] Office Action mailed January 24, 2005.

[3] Office Action mailed January 24, 2005.

Examiner, or that there is a reasonable expectation of successfully combining them to obtain the present invention.

Specifically, Vitti *et al.* discuss a new *in vitro* bioassay for TSH autoantibodies that measures cAMP (cyclic adenosine monophospate) production in Chinese hamster ovary cells transfected with human THSr (CHO-R cells). In this bioassay, stimulation of cAMP production measures hyperthyroidism, while inhibition of cAMP production indicates hypothyroidism. This assay does not discuss a competition with any labeled TSH (as in the present invention), and even suggests that improved sensitivity with respect to the TRAK assay may be obtained with this technique. Thus, Vitti *et al.* provide no suggestion to conduct the TRAK or any other assay using solid phase techniques, but rather teach away from such a procedure in light of the superior sensitivity of their reported cAMP technique.

In addition, although Vitti *et al.* mentions the TRAK assay, this technique is a liquid phase technique which requires precipitation. Furthermore, contrary to the Examiner's remarks, ***no porcine TSH receptor is ever immobilized to a plate in the TRAK assay***. Again, the Examiner can point to no place in Vitti *et al.*, which illustrates the suggestion to the skilled artisan to conduct an assay on the solid phase.

Furthermore, none of the cited references provide any evidence of successfully obtaining the presently claimed invention, let alone its superior results. As evidence of the superior and unexpected nature of the presently claimed invention, the Applicants draw the Examiner's attention to Costagliola *et al.*,[4] which provides a comparison of the conventional radioreceptor assays (*e.g.*, the TRAK assay) and those of the present invention (*e.g.*, the TRAKhuman assay). As can be seen from the abstract "a specificity of 99.6% with a sensitivity of 98.8%" was obtained for the solid state assays, while a "99.6% specificity and 80.2% sensitivity" was obtained for the solution phase assay. Such an enhanced specificity could hardly be expected from the cited art.

Furthermore, the shortcomings of Vitti *et al.* are not remedied by Nicholson or Morgenthaler. These references discuss some monoclonal antibodies obtained by immunizing a mouse with a recombinant full or partial TSH receptor peptide obtained in insect cells. It is well known that recombinant TSH receptor peptides obtained this way do

[4] J. Clin. Endocrinol. Met., 84(1), 90-97 (1999). A copy of this work has been provided for the Examiner's convenience.

not constitute a functional TSH receptor, because they lack, *e.g.*, the ability to bind TSH. The mAb made and tested in these works showed some binding to certain TSH receptor preparations. Being reagents of the same type as TSH receptor autoantibodies, they may be thought of as competitors for autoantibodies to be determined. Thus, although these references could, conceivably, suggest the use of antibodies for detecting TSH receptor autoantibodies in Graves' disease, they hardly suggest the use of autoantibodies to immobilize a functional human TSH receptor preparation in order to design an improved solid phase competitive assay.

Furthermore, in regard to the Examiner's accusation that "the reference [Nicholson] monoclonal antibodies such as A10 and A11 recognize the conformational epitopes of the human TSH receptor, since the antibodies bind only to acetone fixation and not the PLP fixation of the human TSH receptor", the Applicants respectfully disagree. Nicholson discusses both A10 and A11 binding to the same short peptide sequence comprising amino acids 22-35 of the human TSH receptor. Both monoclonal antibodies A10 and A11 are therefore ***sequential*** antibodies, obtained by immunization with an antigen, in which said amino acids apparently are exposed and can act as an antigen. By treatment with acetone, the native receptor is denatured and said sequence becomes accessible.

Finally Applicants note that none of the above shortcomings are addressed by Harlow *et al.* which merely describes general procedural techniques and provides no suggestion or guidance to the skilled artisan to selected specific monoclonal antibodies that recognize only conformational eptiopes of the human TSH receptor obtained by immunizing an animal with a DNA plasmid construct.

Therefore, all objections and rejections having been addressed, it is respectfully submitted that the present application is in a condition for allowance and a Notice to that effect is earnestly solicited.

A Change of Correspondence Address (PTO/SB/122) and a Notice of Appeal with the appropriate fees are being filed concurrently herewith.

Should any issues remain unresolved, the Examiner is encouraged to contact the undersigned attorney for Applicants at the telephone number indicated below in order to expeditiously resolve any remaining issues.

Respectfully submitted,

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Second Generation Assay for Thyrotropin Receptor Antibodies Has Superior Diagnostic Sensitivity for Graves' Disease

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ABSTRACT

Detection of autoantibodies to the TSH receptor (TSH-R) in Graves' disease has found widespread use in clinical routine and is performed mostly by commercial RRAs measuring TSH binding inhibitory activity. We report in this study on a second generation TSH binding inhibitory assay using the human recombinant TSH-R with two major improvements: 1) superior diagnostic sensitivity for Graves' disease, and 2) for the first time, nonradioactive and radioactive coated tube (CT) technology. Full-length human recombinant TSH-R was expressed in the K562 leukemia cell line and grown in suspension at a high density. A murine monoclonal antibody was selected for binding to the native TSH-R without interfering with autoantibodies or TSH and was coated to polystyrene tubes. After detergent extraction, TSH-R was affinity immobilized on antibody-coated tubes. The binding of TSH to the TSH-R could be demonstrated by the addition of ^{125}I- or acridinium ester-labeled bovine TSH, and this binding could be inhibited by sera from patients with Graves' disease up to 95%. Subsequently, these novel assays, a CT RRA and a CT luminescence receptor assay, were compared to the conventional RRA based on porcine antigen in a blinded clinical multicenter trial. Sera from 328 patients with Graves' disease (86 untreated, 116 treated, and 126 in remission) and 520 controls (comprised of healthy blood donors and patients with autoimmune diseases or goiter) were tested in all 3 assays. Receiver-operating characteristic plot analysis resulted in a specificity of 99.6% with a sensitivity of 98.8% for both CT assays, compared to 99.6% specificity and 80.2% sensitivity for the conventional RRA ($P < 0.001$). In all 3 groups of patients with Graves' disease, the 2 CT assays were significantly more sensitive for the disease than the conventional assay, without loss of specificity in the control groups. This increase in sensitivity and the nonradioactive or radioactive CT format constitute a significant improvement over the currently available assays. (*J Clin Endocrinol Metab* **84:** 90–97, 1999)

THE PATHOGENETIC role of autoantibodies to the TSH receptor (TRAb) in sera of patients with autoimmune thyroid disease has been clearly established (see Refs. 1 and 2 for review). At present, autoantibodies are essentially being detected by two kinds of assays (see Ref. 3 for review). The commercially available RRAs measure autoantibodies described by their TSH binding inhibitory (TBII) activity. Alternative detection systems measure the production of cAMP in response to a TSH-R interaction with stimulating antibodies (TSAb) or blocking antibodies (TBAb) (4–8). Although those bioassays have recently been modified to suit routine laboratories (9, 10), the necessity of using cell lines and the need for tissue culture facilities still limit their use outside specialized centers.

Other detection systems have been described, such as autoantibody detection by FACS (11, 12), immunoprecipitation (13), immunocytochemistry (14), or transferring serum to nude mice (15). These methods are still in an experimental state and are too time consuming and cumbersome for everyday use.

The only validated routine assays for the detection of TRAb are RRAs using porcine thyroid membrane extracts based on the method of Shewring and Rees Smith (16). In their commercialized form, these assays are widely used. Although about 70–90% of TRAb in the serum of Graves' disease patients are detected by these RRA, there is evidence of clinical hyperthyroid patients classified as Graves' disease who are negative in the RRA using porcine-derived antigen (17, 18). To increase the sensitivity of the assay, many groups have tried to replace the porcine source of TSH receptor by human recombinant antigen. This approach was reported to work on a small scale (19, 20), but the difficulties associated with the large scale production of human TSH receptor have

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limited its applicability in clinical practice. These early experiments were performed with mammalian cell lines, which produced a well glycosylated TSH-R that binds TSH and autoantibodies efficiently. However, the growth conditions of these cells and the level of receptor expression did not permit the production of sufficient antigen for routine assays. Alternative approaches by overexpressing TSH-R in bacteria (21, 22) or insect cells (23–25) did not lead to the expression of a functional receptor, *i.e.* binding both TSH and autoantibodies.

Here we show that expression of the human TSH-R (hTSH-R) in the leukemia cell line K562 yielded adequate amounts of bioactive human antigen. In addition, a monoclonal antibody (moAb) (26) recognizing only the native receptor has allowed for the development of a new RRA in the coated tube (CT) format as well as a nonradioactive chemiluminescence assay. The clinical superiority of these assays compared to the conventional RRA was demonstrated with 328 sera from patients with Graves' disease and 520 different controls.

Subjects and Methods

Patients

Included in the study were 328 patients with Graves' disease. Graves' disease was diagnosed initially according to standard clinical criteria (suppressed TSH, elevated T_3 or free T_4, goiter, sonography, and signs of Graves' ophthalmopathy when present). Patients were grouped according to their metabolic state in patients with active hyperthyroid Graves' disease without or with less than 4 weeks of treatment with antithyroid drugs (group 1, n = 86), patients receiving treatment for longer than 4 weeks (group 2, n = 126), and patients in remission without treatment (group 3, n = 116).

Furthermore, we included 54 patients with Hashimoto's thyroiditis (diagnosed on the bases of clinical hypothyroidism, sonography, and the presence of antithyroid peroxidase and/or antithyroglobulin autoantibodies; group 4), 69 patients with nonthyroid autoimmune diseases (type I diabetes, rheumatoid arthritis, and systemic lupus erythematosus) with nonthyroid autoantibodies (group 5), 115 patients with goiter but no signs of autoimmune thyroid disease (group 6), and 282 healthy individuals without a history of thyroid disease who were euthyroid and negative for antithyroid peroxidase and antithyroglobulin autoantibodies (group 7). The clinical data of the patients are summarized in Table 1.

All sera were collected between 1997 and 1998 prospectively at the centers participating in the study, coded, and stored at −20 C. All antibody detections were performed in a blinded assay design at the research laboratories of B.R.A.H.M.S Diagnostica (Berlin, Germany).

Generation of recombinant TSH-R-producing cells

The coding sequence of the hTSHR complementary DNA (27) was subcloned into the *KpnI/XbaI* sites of pEFIN, a bicistronic vector developed at EUROSCREEN (Brussels, Belgium) (28). K562 cells were maintained at 37 C and 5% CO_2 in DMEM containing 10% FCS. For transfection, 2×10^6 exponentially growing cells were electroporated at 0.2 kV/cm, 960 μF (Bio Rad Gene Pulser) for 25 ms in the presence of 20 μg specific linearized plasmid DNA and seeded into culture flask. Forty-eight hours after electroporation, selection was started with 800 μg/mL G418 (Life Technologies, Grand Island, NY). After cloning by limiting dilution, a stable clone expressing high levels of hTSHR was selected. The number of receptors expressed per cell and their dissociation constant (K_d) were computed from displacement curves in which binding of ^{125}I-labeled bovine TSH (bTSH) was competed for by increasing concentrations of unlabeled bTSH. It was estimated that the cells harbored about 1×10^6 receptors with a K_d of 4.8 mIU/mL.

Selection of moAb by FACS

K562 cells expressing hTSHR or K562 cells (wild type) were transferred into Falcon 2052 tubes (200,000 cells/tube; Becton Dickinson, Mountain View, CA). Cells were centrifuged at 500 × *g* at 4 C for 3 min, and the supernatant was removed by inversion. They were incubated for 30 min at room temperature with 100 μL phosphate-buffered saline (PBS)-0.1% BSA containing 10 μL culture supernatant from 3 different hybridomas (BA8, 3G4, 5A6) (26). The cells were washed with 4 mL PBS-0.1% BSA and centrifuged as above. They were incubated for 30 min on ice in the dark with fluorescein-conjugated γ-chain-specific goat antimouse IgG (Sigma Chemical Co., St. Louis, MO) in the same buffer. Propidium iodide (10 μg/mL) was used for detection of damaged cells that were excluded from the analysis. Cells were washed once again and resuspended in 250 μL PBS-0.1% BSA. The fluorescence of 5,000 cells/tube was assayed by a FACScan flow cytofluorometer (Becton Dickinson and Co., Eerenbodegem, Belgium). The murine moAb BA8 bound specifically to the native human TSH-R (26) and was used for subsequent studies.

Preparation of hTSH-R

K562 cells expressing the hTSH-R (K562-TSH-R) were grown in Spinner flasks at 37 C and 5% CO_2 in DMEM containing 10% FCS (without G418) to a density of 1.5×10^6 cells/mL. Cells were harvested by centrifugation (2,400 × *g*, 10 min, 4 C), washed once with ice-cold PBS, and stored at −80 C. Frozen cells were resuspended in washing buffer [50 mmol/L HEPES (pH 6.8) and 50 mmol/L NaCl] and centrifuged (100,000 × *g*, 30 min, 4 C). The cell pellet was resuspended in extraction buffer [100 mmol/L HEPES (pH 6.8), 2% Triton X-100, and Complete protease inhibitors (Boehringer Mannheim, Mannheim, Germany)] and homogenized in a Potter homogenizer (Braun, Melsungen, Germany). After centrifugation (100,000 × *g*, 30 min, 4 C), BSA (protease free, Sigma Chemical Co.) was added to a final concentration of 1% to the supernatant containing the solubilized TSH-R.

Production of TSH-R coated tubes

The moAb BA8 (26) was coated for 20 h on polystyrene tubes (1.5 μg/tube) in 0.3 mL buffer [10 mmol/L Tris-HCl (pH 7.5) and 50 mmol/L NaCl]. Tubes were blocked with 10 mmol/L sodium phosphate buffer containing 3% Karion FP and 0.5% protease-free BSA (Sigma Chemical Co.), pH 6.8. The TSH-R-containing extract was diluted 1:50 and added to the tubes. Affinity binding of the TSH-R to the antibody was performed at 4 C for 20 h. Tubes were blocked again [50 mmol/L HEPES (pH 6.5), 0.25% Triton X-100, 1% Karion FP, and 0.5% BSA] and lyoph-

TABLE 1. Clinical data for the patients in the different groups

Group no.	n	Patients with Graves' disease and controls	Age (yrs)	Gender (female:male)	Basal TSH [mU/L (0.3–4.0)]	T_3 [μg/L (0.8–1.8)]	fT_4 [ng/L (8–18)]
1	86	Untreated Graves' disease	40 (13–80)	6.4:1	0.01 (0.01–0.20)	2.6 (0.8–10.6)	23.3 (7.7–87.0)
2	126	Treated Graves' disease	40 (20–80)	6.7:1	0.08 (0.01–22.0)	1.5 (0.6–4.8)	11.3 (3.9–64.5)
3	116	Graves' disease in remission	44 (19–76)	4.9:1	1.15 (0.01–39.3)	1.2 (0.6–2.1)	12.4 (4.0–20.0)
4	54	Hashimoto's thyroiditis	44 (25–88)	8.6:1	1.30 (0.01–46.5)	1.2 (0.7–2.7)	12.4 (8.0–53.6)
5	69	Nonthyroid autoimmune disease	38 (17–74)	1.6:1	1.10 (0.20–6.30)	1.1 (0.7–2.1)	12.0 (8.0–19.0)
6	115	Nonautoimmune thyroid disease	58 (19–80)	3.6:1	0.90 (0.01–44.0)	1.2 (0.6–3.1)	12.7 (2.0–27.0)
7	282	Healthy controls	45 (20–73)	0.7:1	1.30 (0.20–4.40)	1.1 (0.6–1.9)	12.0 (8.0–18.0)

Values given are medians, with the range in *parentheses.*

ilized. Ten liters of culture medium resulted in the preparation of 20,000 coated tubes.

Preparation of labeled bTSH

bTSH was affinity purified from bovine pituitaries (final activity, 50–60 TSH IU/mg protein) and labeled with ^{125}I using the chloramine-T method, yielding a specific activity of 58 μCi/μg protein. Acridinium ester-labeled bTSH was produced as follows. bTSH (100 μg; 50–60 TSH IU/mg protein) in 20 mmol/L sodium phosphate buffer, pH 7.0, was incubated for 15 min at room temperature with 10 μL acridinium ester (1 mg/mL in acetonitril; Hoechst AG, Frankfurt, Germany). Labeled bTSH was purified by high performance liquid chromatography using a Waters-Protein Pak SW 125 column (running buffer, 0.1 mol/L ammonium acetate, pH 5.5; flow rate, 0.6 mL/min).

Autoantibody measurement in conventional TBII assay

Autoantibody measurement was performed with a commercial RRA (TRAK-Assay, B.R.A.H.M.S Diagnostica) following the manufacturer's instructions. The functional assay sensitivity of this RRA is 8 U/L.

Autoantibody measurement in CT TBII assays

Patients' samples or standards (100 μL) were added in duplicate to hTSH-R coated tubes. To this were added 200 μL buffer containing 100 mmol/L HEPES, 20 mmol/L ethylenediamine tetraacetate, 0.5 mmol/L N-ethyl-maleimide, 0.1 mmol/L leupeptin, 1% BSA, 0,5% Triton X-100, and 5 μg antihuman TSH antibody (Sigma Chemical Co.), pH 7.5. After 2-h incubation under shaking (300 U/min) at room temperature, tubes were washed once with 2 mL washing buffer. Then, 200 μL tracer were added containing either ^{125}I- or acridinium ester-labeled bTSH (1 ng/tube; B.R.A.H.M.S Diagnostica), followed by 1-h incubation at room temperature. Tubes were washed twice with 2 mL washing buffer, and detection was performed in either a γ-counter or a luminometer.

Calibration of CT assays

To compare data between individual test runs, all raw data [counts per min for radioligand assay, relative light units (rlu) for chemiluminescence assay] were expressed in TRAK units as calculated from a standard curve that was included in every run. To obtain a representative standard curve, 20 sera from Graves' disease patients with high titers were pooled, diluted, and calibrated using the TRAK-Assay.

The Medical Research Council standard of 1966 (long acting thyroid stimulator, lot 65/122) and the WHO standard of 1995 (TSAb, lot 90/672) were tested in both assays. One Medical Research Council unit resulted in approximately 1.5 TRAK units, and 1 WHO unit resulted in 4 TRAK units, respectively.

Definition of cut-off and statistical analysis

To obtain the optimal decision threshold level for positivity, receiver operating characteristic (ROC) analysis was performed (29). Sensitivity/specificity pairs were calculated by varying the decision threshold levels over the entire range of TRAK units. The sensitivity (true positive results) of all three assays was calculated from the 86 patients in group 1 (untreated Graves' disease). On the other side, the specificity (true negative results) was calculated from 282 healthy blood donors (group 7). The experimental cut-off was determined for all assays at 99.6% specificity. Statistical analysis was performed using χ^2 test with Yates correction for comparison of the autoantibody prevalence between the different assays within the respective groups and Mann-Whitney rank sum analysis for comparison of the autoantibody levels in the different groups determined with one assay. Correlation analysis was performed with Pearson correlation. For method comparison of CT RRA and TRAK assay, the nonparametric approach according to Passing and Bablok was used (30).

Results

The assay described here is based on the use of two renewable reagents: the hTSH-R stably expressed in a high yield eukaryotic cell/vector system and a moAb (BA8) recognizing the native hTSHR without interfering with binding of TSH or TRAb. The generation of the moAb BA8 by genetic immunization has been described previously (26). The K562 leukemic cell line was chosen because it grows easily at high density in suspension. The cells were transfected with a bicistronic vector harboring the hTSH-R complementary DNA and the neomycin resistance gene. This ensures that the clones resisting G418 selection will keep the expression of the TSH-R (28). One cell line was selected for its particularly high level of expression of the receptor, as judged from flow cytometry experiments using moAb BA8 (Fig. 1B). Competition binding experiments with a [^{125}I]bTSH tracer indicated that the cell line expresses about 10^6 receptors/cell with a K_d of 4.8 mIU/mL (Fig. 1A).

Large amounts of hTSH-R were purified by detergent extraction. The moAb BA8 was coated on polystyrene tubes in a concentration of 1.5 μg/tube. As this antibody binds only native TSH-R in the correct conformation and does not interact with the binding sites of TSH, TBII, TSAb, or TBAb on the TSH-R (26), it provides an excellent capture antibody for solid phase assays for the detection of autoantibodies to the TSH-R. After affinity immobilization of the recombinant TSH-R, the tubes were lyophilized and stored at 4 C. The binding of TSH to the TSH-R could be demonstrated by the addition of 1 ng ^{125}I-labeled bTSH/tube [2 kBq (0.08 μCi) activity]. After washing, 8–12% of the total TSH added was bound specifically to TSH-R-coated tubes, but only 0.1–0.2% was bound to tubes coated with BA8 alone. This binding of TSH to the TSH-R could be inhibited by sera from patients with Graves' disease up to 95% of the binding obtained in the

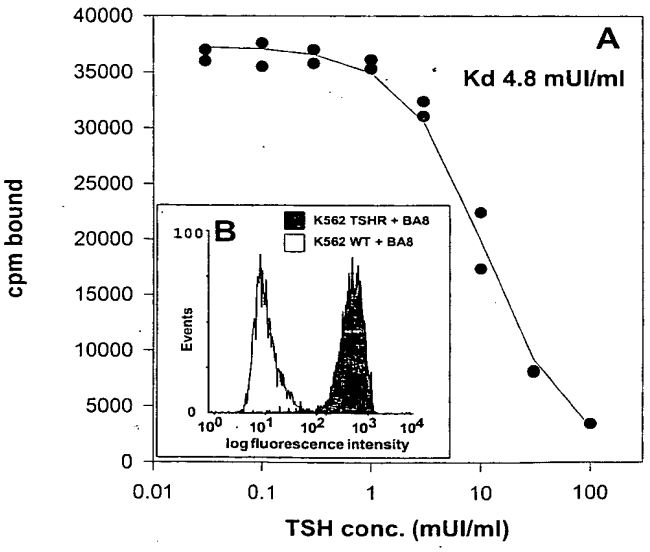

FIG. 1. A, The number of receptors expressed per K562 cells and their dissociation constant (KdS) were computed from displacement curves in which binding of ^{125}I-labeled bovine TSH was competed for increasing concentrations of unlabeled bovine TSH. It was estimated that the cells harbored about 1×10^6 receptors with a K_d of 4.8 mIU/mL. B, FACS analysis of K562 cells expressing the human TSH-R (*gray*) or K562 cells (wild type) with the murine anti TSH-R monoclonal antibody BA8. The specific binding of BA8 to the native TSH-R is demonstrated by the shift in fluorescence intensity.

presence of control serum. Addition of excess unlabeled TSH gave a similar reduction. Figure 2 shows representative [^{125}I]TSH binding in the presence of different sera and unlabeled TSH. Similar results were obtained using the acridinium ester-labeled bTSH in chemiluminescence detection (data not shown).

To standardize the system, allowing for the detection of a large number of sera measured in different runs, data were not expressed in counts per min or relative light units but in TRAK units per L calculated from a standard curve. In contrast to conventional TBII assays, where unlabeled bTSH in the standards mimics the effect of antibodies in competing for labeled bTSH, we decided to use a homogeneous system with autoantibodies for standardization. For both the CT RRA and the CT luminescence receptor assay (LRA), a pool of highly positive sera from patients with Graves' disease was diluted serially and expressed as a ratio of B/Bo, were B is the binding of the sample, and Bo is the binding of the negative control serum. Calibration with respect to the standard curve of the conventional TRAK assay resulted in standards ranging from 3.5–224 U/L. Figure 3 shows individual sera and a representative standard curve of pooled sera for the CT LRA and CT RRA. Measuring the Medical Research Council long acting thyroid stimulator standard (see *Materials and Methods*) and the WHO TSAb standard, 1 Medical Research Council U/L resulted in 1.5 TRAK U/L, and 1 WHO U/L resulted in 4 TRAK U/L, respectively, in both assays.

To assess the technical sensitivities of the CT assays [also called the functional assay sensitivity (FAS)], as defined by an interassay coefficient of variation less than 20% (31), we measured sera covering the entire range of the standard curve in 10 individual runs. The coefficient of variation of each sample value and the deducted FAS at 3.0 U/L is shown in Fig. 4 for the CT RRA. The FAS for the CT LRA has the same value (data not shown).

To define the clinical cut-off for a positive serum with autoantibodies to the TSH-R, we performed ROC plot analysis of the data from the patients in group 1 (active Graves' disease) and group 7 (healthy controls) for all three assays. Figure 5 shows a plot of the sensitivity and specificity of all three assays. With a specificity of 99.6% at a cut-off of 4 U/L, the sensitivity of both CT assays was 98.8% compared to 99.6% specificity and 80.2% sensitivity of the conventional system at a cut-off of 11 U/L. Using this cut-off for all groups of patients the sensitivity of the new assays compared to that

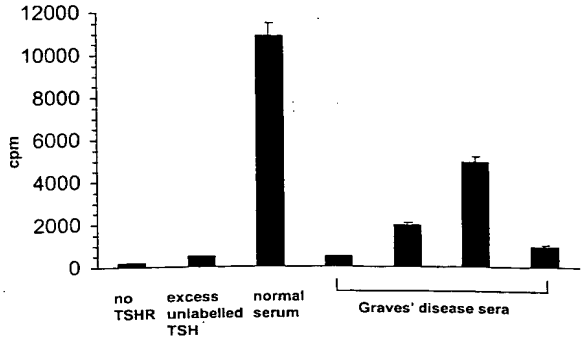

FIG. 2. Binding of ^{125}I-labeled bTSH (100,000 cpm total activity) to TSH-R on tube. About 8–12% of the total activity is bound specifically to the TSH-R in the presence of normal serum. In the presence of sera from patients with Graves' disease, this binding is reduced up to 95%. The same effect is seen with excess unlabeled bTSH. The nonspecific binding to the tubes without TSH-R is between 0.1–0.2% of total activity. Similar results were obtained using acridinium ester-labeled bTSH (not shown).

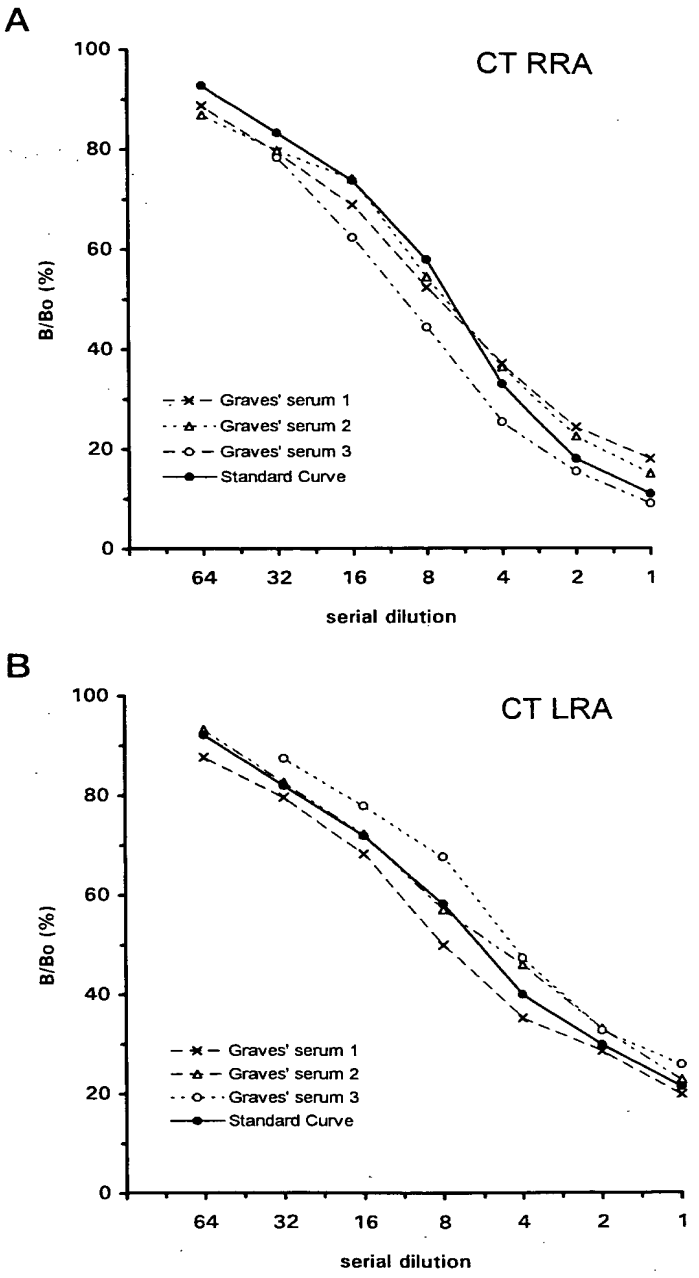

FIG. 3. A pool of 20 positive sera was diluted serially and expressed as a ratio of B/Bo, were B is the binding of the sample, and Bo the binding of the negative control serum. Calibration at the standard curve of the TRAK assay resulted in standards ranging from 3.5–224 U/L. Shown are individual sera and the pooled standard curve for the CT LRA and CT RRA.

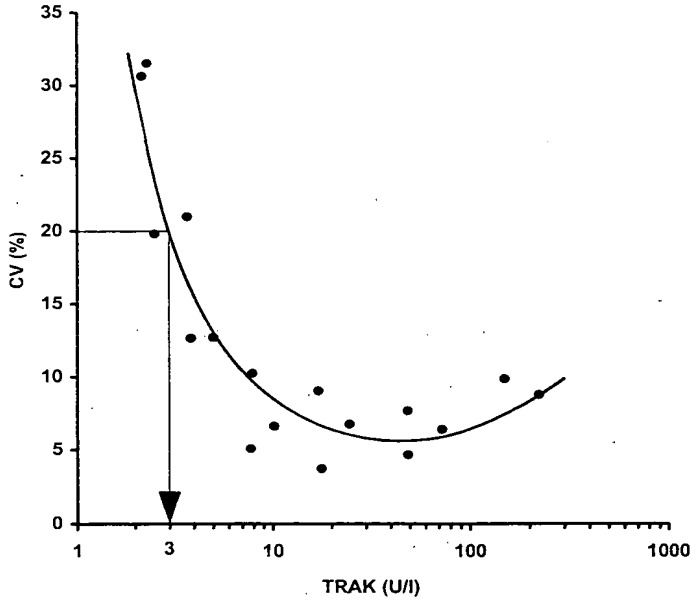

FIG. 4. FAS of the CT RRA, as defined by an interassay coefficient of variation (cv) less than 20%. Sera covering the entire range of the standard curve were measured in 10 individual runs, and the cv (percentage) of each sample is shown. The FAS for the CT RRA is shown as 3.0 U/L.

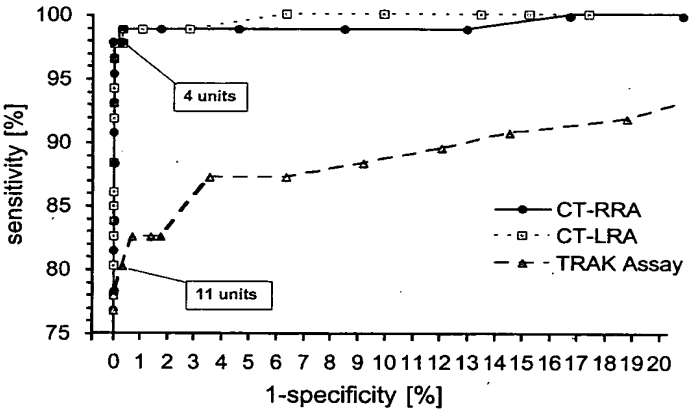

FIG. 5. To obtain the optimal decision threshold level for positivity, ROC analysis was performed. Sensitivity/specificity pairs were calculated by varying the decision threshold levels over the entire range of units per L. The cut-off used for the clinical evaluation is indicated by the *boxes*.

of the conventional assay is shown in Table 2. In all three groups of patients with Graves' disease (groups 1–3), the CT assays were significantly more sensitive for the disease than the conventional assay (by χ^2 test with Yates correction: group 1, $\chi^2 = 14.0$, $P < 0.001$; group 2, $\chi^2 = 4.1$, $P < 0.05$; group 3, $\chi^2 = 4.0$, $P < 0.05$). In contrast, there was no significant difference between the assays in the control groups 5, 6, and 7. Although the CT assays detected about 10% more patients in group 4 (Hashimoto's thyroiditis) than the conventional RRA, the difference was not significant ($\chi^2 = 1.62$; $P = 0.23$).

The distribution of the autoantibody levels in groups 1–7 is shown as box and whisker plots in Fig. 6 for all three assays. In all three assays there were significantly higher autoantibody levels in the groups with Graves' disease than in all control groups, including group 4 ($P < 0.0001$, by Mann-Whitney rank sum test). Group 1 (untreated Graves' disease) and group 2 (treated Graves' disease) showed no difference in the conventional RRA ($P = 0.65$), although there was a stronger (but still not significant) difference in the CT assays between those groups ($P = 0.08$). However, in all three assays, groups 1 and 2 had significantly higher autoantibody levels than group 3 (Graves' patients in remission; $P < 0.0001$). Neither assay showed a significant difference in the autoantibody levels in the healthy controls (group 7), patients with nonthyroid autoimmunity (group 5), or patients with nonautoimmune thyroid disease (group 6), but all three assays showed a significant difference between the Hashimoto's thyroiditis patients (group 4) and the healthy controls ($P < 0.0001$). Although patients and controls were from two centers, there was no heterogeneity, *i.e.* no differences within the groups between the two centers were observed.

A nonparametric method comparison between the CT RRA and the conventional RRA according to Passing and Bablok (30) revealed a biased relationship toward the CT RRA (slope: 1.29; 95% confidence interval: 1.18–1.43; intercept, −4.99; 95% confidence interval: −7.06 to −2.74). However, there was a strong positive correlation between the data obtained in the conventional RRA and those obtained in the CT RRA as shown in Fig. 7 ($r = 0.91$; $P < 0.001$).

Discussion

In the present clinical setting, TSH-R antibodies are assayed in patients with the various forms of thyrotoxicosis to identify those with Graves' disease. Their treatment differs from other forms of hyperthyroidism, as a considerable proportion of these patients undergo long term remission after antithyroid drug treatment and can therefore be spared ablative forms of therapy. As TRAb titers decrease in the course of antithyroid drug treatment, their rise may herald an early relapse of hyperthyroidism. Nevertheless, despite evidence in favor of a predictive value of continuously high TRAb levels for relapsing Graves' disease (2, 32), prospective and multicenter studies to date had to rely on assays using porcine antigen.

Routine use of recombinant hTSH-R in TBII assays has not been practical until now because the high yield production systems, like bacteria (21, 22) or baculovirus (23–25), did not produce bioactive receptor (*i.e.* showing TSH binding). The available CHO cell lines (7, 11, 12, 33) do produce bioactive receptor, but their growth conditions for large scale production are fastidious. The K562 line described in the present study provides an efficient solution to this problem; it grows in suspension to densities of 1.5×10^6 cells/mL and expresses stably about 10^6 receptors/cell.

Our results demonstrate a close to 100% sensitivity of the second generation TBII assay in hyperthyroid patients with Graves' disease. Thus, this assay using recombinant hTSH-R is clearly superior to its predecessor based on porcine thyroid membrane preparations. The improved sensitivity for Graves' disease from 80% to nearly 100% may be due to

TABLE 2. TSH-R autoantibody median concentration, range, and prevalence in the different patient groups, measured in the new CT RRA, CT LRA, and conventional TBII assay (TRAK assay)

	CT RRA	CT LRA	TRAK assay
Group 1: untreated Graves' disease			
Median (U/L)	37.6	44.9	26.9
Range (U/L)	1.0–532.0	3.0–603.0	0.0–1139.0
Positive (total)	85 (86)	85 (86)	69 (86)
Prevalence (%)	98.8	98.8	80.2
χ^2 test (P)	<0.001	<0.001	
Group 2: treated Graves' disease			
Median (U/L)	34.8	34.3	26.4
Range (U/L)	0.0–1328.4	0.0–1370.0	0.0–1321.0
Positive (total)	115 (126)	115 (126)	103 (126)
Prevalence (%)	91.3	91.3	81.7
χ^2 test (P)	<0.05	<0.05	
Group 3: Graves' disease in remission			
Median (U/L)	3.8	3.8	7.9
Range (U/L)	0.0–389.0	0.0–344.0	0.0–250.5
Positive (total)	56 (116)	57 (116)	40 (116)
Prevalence (%)	48.3	49.1	34.5
χ^2 test (P)	<0.05	<0.05	
Group 4: Hashimoto's thyroiditis			
Median (U/L)	1.1	0.0	6.8
Range (U/L)	0.0–38.4	0.0–35.9	0.0–15.7
Positive (total)	8 (54)	8 (54)	3 (54)
Prevalence (%)	14.8	14.8	5.6
χ^2 test (P)	0.20	0.20	
Group 5: nonthyroid autoimmune disease			
Median (U/L)	0.0	0.2	3.5
Range (U/L)	0.0–6.0	0.0–6.9	0.0–15.1
Positive (total)	2 (69)	2 (69)	1 (69)
Prevalence (%)	2.9	2.9	1.4
χ^2 test (P)	NS	NS	
Group 6: nonautoimmune thyroid disease			
Median (U/L)	0.0	0.3	4.9
Range (U/L)	0.0–8.3	0.0–9.7	0.0–14.5
Positive (total)	3 (115)	3 (115)	1 (115)
Prevalence (%)	2.6	2.6	0.9
χ^2 test (P)	NS	NS	
Group 7: healthy controls			
Median (U/L)	0.0	0.0	4.4
Range (U/L)	0.0–5.2	0.0–5.2	0.0–11.3
Positive (total)	1 (282)	1 (282)	1 (282)
Prevalence (%)	0.4	0.4	0.4
χ^2 test (P)	NS	NS	

several properties. First, there may be a better configurational accessibility of the recombinant hTSH-R preparation for its antibodies that is not present on the crude porcine membrane preparation. Second, solid phase technology allows for a reduction in nonspecific binding. The better signal to noise ratio leads to a lower decision threshold for the cut-off and increases the sensitivity, as shown by ROC plot analysis. For clinical purposes this high sensitivity allows a rapid distinction between autoimmune and other forms of hyperthyroidism and could obviate the need for other diagnostic procedures in thyrotoxic patients positive for TRAb. Thus, the new assay represents an improvement in the management of Graves' disease.

Currently, TRAb are measured at the beginning and during the course of antithyroid drug treatment to detect early relapses. A large multicenter study (34), among others, has confirmed that patients with elevated TRAb at the end of drug treatment have a significantly higher relapse rate than those without. However, the low sensitivity and specificity of the assay precluded its use in the prediction of the individual clinical course (34). A meta-analysis combining studies of relapse prediction in Graves' disease later showed again that TRAb activity is clearly associated with relapse. However, a considerable proportion of TRAb-positive or -negative patients was found in the remission or relapse groups, respectively (35). These studies were based on porcine membrane preparations; consequently, a prospective evaluation of the new assay is warranted that evaluates its predictive value for relapse or remission in the long term course of Graves' disease.

The median levels of TRAb titers were significantly higher in patients with active Graves' disease with or without antithyroid drug treatment than in those in remission, although the latter still contained about 48% TRAb positives (compared with 35% of TRAb-positive patients in the porcine membrane assay). Similarly, 15% of patients with Hashimoto's thyroiditis had detectable TRAb titers using the new CT assays in contrast to 6% with the conventional assay. These TRAb levels, although measurable, are functionally different, as they are not associated with clinical or biochemical hy-

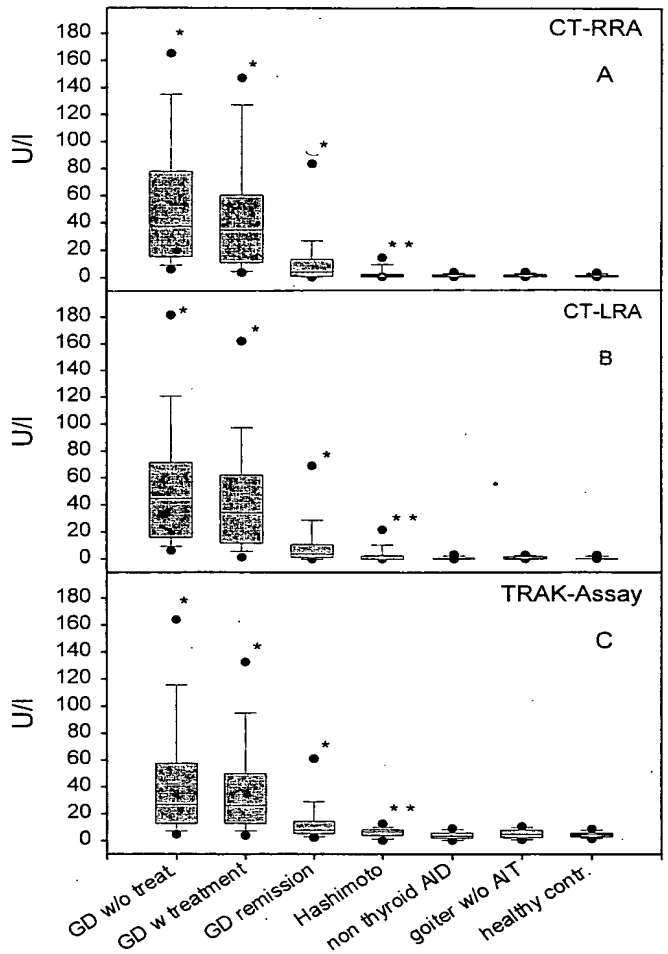

FIG. 6. Distribution of autoantibodies in different groups of patients shown as box plots, indicating 25–75th percentiles (*box*) with median (*line*), 10–90th percentile (*whiskers*), and 5–95th percentile (*dots*). A, CT RRA; B, CT LRA; C, conventional RRA (TRAK assay). GD, Graves disease; Hashimoto, Hashimoto's thyroiditis; AID, autoimmune disease; AIT, autoimmune thyroid disease. *, $P < 0.0001$ *vs.* all control groups; **, $P < 0.001$ *vs.* nonthyroid AID, goiter, and healthy control groups (by Mann-Whitney rank sum analysis).

perthyroidism. They may have binding and blocking capabilities in contrast to the stimulating Igs found in active Graves' disease. Autoantibodies that block the binding of TSH to its receptor may even lead to hypothyroidism, as in certain cases of atrophic thyroiditis (primary myxedema). Such functional differences can only be distinguished when comparing the binding inhibition assay with the measurement of stimulating Igs (4–10).

Low TRAb levels were also detected in a limited number of individuals belonging to the control groups (1 of 282 healthy controls, 3 of 115 in nonautoimmune thyroid disease patients, and two out of 69 with nonthyroid autoimmune disease). The significance of these positive values remains to be determined. A close follow-up of these individuals will reveal whether the new TBII assays will be useful for the early detection of autoimmune hyperthyroidism.

Although quantitative correlation between TSAb and TBII

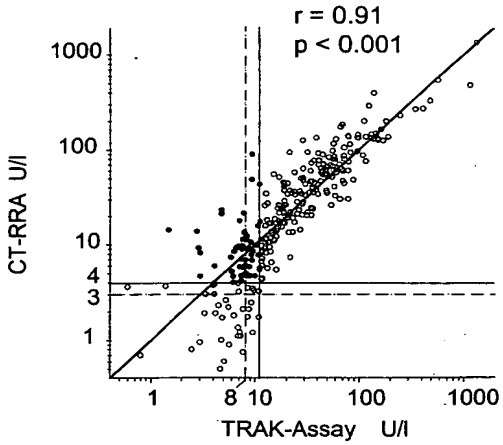

FIG. 7. Correlation (r = 0.91; $P < 0.001$) of the CT RRA and the conventional RRA. The ideal correlation is indicated by the *diagonal line*. According to the method comparison of Passing and Bablok (30), the data are biased toward the CT RRA. The FAS is shown by the *dotted line*; the clinical cut-off is shown by the *solid line*. Sera positive in the CT RRA, but negative in the conventional assay, are indicated by *closed circles*.

assays is relatively poor (10), only a very small number of patients who are clearly TSAb-positive score negative in current TBII assays (10). This, which constitutes the main justification for the routine clinical use of TBII assays, may indicate that stimulation of the receptor by TSAbs implies recognition of an epitope(s) that is part of the TSH-binding site; alternatively, it is compatible with the coexistence in Graves' patients of stimulating antibodies (that may not always be endowed with TBII properties) and antibodies that compete for TSH binding (and may not be endowed with TSAb or TBAb properties) (36).

Identification of the epitopes corresponding to the various autoantibody categories is a major focus of current research (37). The view that TSAbs and TBAbs would recognize distant parts of the TSH-R ectodomain (36) will need experimental confirmation by the isolation of the individual TRAbs from patient's serum. Similarly, the respective importance of the nature of autoantibodies or intrinsic/paracrine thyroid factors to explain the variability in goiter size in Graves' patients remains to be defined.

It is hoped that future generations of TRAb assays will allow for the measurement of the spectrum of autoantibodies with specific functional characteristics. In the meantime, with their increased sensitivity, we consider that the new TBII assays described here constitute a significant improvement over the currently available assays and deserve evaluation in additional prospective studies.

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